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Regulation of white and opaque cell type formation in *Candida albicans* by Rtt109 and Hst3

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Summary

How different cell types with the same genotype are formed and heritability maintained is a fundamental question in biology. We utilized white-opaque switching in *Candida albicans* as a system to study mechanisms of cell type formation and maintenance. Each cell type has tractable characters, which are maintained over many cell divisions. Cell type specification is under the control of interlocking transcriptional feedback loops, with Wor1 being the master regulator of the opaque cell type. Here we show that deletion of *RTT109*, encoding the acetyltransferase for histone H3K56, impairs stochastic and environmentally stimulated white-opaque switching. Ectopic expression of *WOR1* mostly bypasses the requirement for *RTT109*, but opaque cells lacking *RTT109* cannot be maintained. We have also discovered that nicotinamide induces opaque cell formation, and this activity of nicotinamide requires *RTT109*. Reducing the copy number of *HST3*, which encodes the H3K56 deacetylase, also leads to increased opaque formation. We further show that the Hst3 level is down regulated in the presence of genotoxins and ectopic expression of *HST3* blocks genotoxin induced switching. This finding links genotoxin induced switching to Hst3 regulation. Together these findings suggest *RTT109* and *HST3* genes play an important role in the regulation of white-opaque switching in *C. albicans*.

Introduction

Epigenetic regulation is fundamental for adaptation to environmental changes and cell specialization/differentiation. Epigenetic states are heritably maintained, allowing cells to "remember" past changes of the external environment or developmental cues. Understanding molecular mechanisms underlining epigenetic states is important for regenerative medicine and cancer treatment. Despite this importance, studies of cell fate specialization in higher eukaryotes are often hindered by a high degree of heterogeneity and sensitivity to a multitude of extrinsic signaling. An alternative, but unicellular eukaryotic system of epigenetic inheritance of specialized cell types is seen in *Candida albicans*, the most significant human fungal pathogen. *C. albicans* is able to reversibly switch between two visibly different cell types: white and opaque (Slutsky *et al.*, 1987). White and opaque cells differ in cell shape, the genes they express (Lan *et al.*, 2002, Tsong *et al.*, 2003, Tuch *et al.*, 2010), their mating behaviors (Miller & Johnson, 2002), and virulence properties (Lohse & Johnson, 2008, Kvaal *et al.*, 1999, Morschhauser, 2010). Switching is stochastic and infrequent, with a switching frequency about 10^{-4} per cell division (Rikkerink et al., 1988).

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Certain conditions have been reported to increase the switching frequency. Genotoxic stress is long known for promoting the white to opaque switch (Alby & Bennett, 2009, Morrow *et al.*, 1989). Recently, incubation of white cells under anaerobic conditions, high CO_2 level or N-acetylglucosamine was shown to induce switching to the opaque cell type (Huang *et al.*, 2009, Ramírez-Zavala *et al.*, 2008, Huang *et al.*, 2010). The opaque phase is sensitive to temperature. Upon shifting the growth temperature from room temperature to 37°C, opaque cells switch en masse to white cells (Bergen et al., 1990). The ease of cell culturing and efficient homologous recombination for gene manipulation in *C. albicans* make the white-opaque switching system one of the most tractable eukaryotic systems for studying epigenetic regulation in a biomedical relevant context.

Different cell types are determined by specific gene expression programs, which are controlled by transcription factors and chromatin regulators. White and opaque cell type specification in *C. albicans* is under the control of interlocking transcriptional feedback loops with Wor1 being a master regulator of the white-opaque switch (Huang *et al.*, 2006, Srikantha *et al.*, 2006, Zordan *et al.*, 2006, Zordan *et al.*, 2007). Deletion of *WOR1* blocks opaque cell type formation, whereas ectopic expression of *WOR1* switches white cells to opaque. *WOR1* is preferentially expressed in opaque cells and the positive feedback regulation of *WOR1* expression from Wor1 and another transcription factor, Wor2, is believed to provide the bistable expression of *WOR1* that is essential for the existence of the stable opaque cell type. Despite the large evolutionary distance between mammals and fungi, transcriptome analysis combined with Wor1 ChIP-chip suggests that the Wor1 circuit shares several characteristics with the transcriptional circuits for pluripotency of mammalian embryonic stem cells (Tuch *et al.*, 2010, Zordan et al., 2007). In addition to the transcriptional loops, histone-modifying enzymes constitute another layer of regulation for the white and opaque cell types. Lack of the histone deacetylases HDACs *HDA1* and *RPD3* modify frequencies of switching (Srikantha *et al.*, 2001). More recently, a comprehensive deletion analysis focusing on catalytic subunits of histone-modifying enzymes identified several enzymes that can modulate frequencies of switching (Hnisz *et al.*, 2009). Epistasis analysis mapped them into at least two independent pathways in relation to the known transcriptional regulators, and they all seem to act upstream of *WOR1* (Hnisz *et al.*, 2009).

Acetylation within the globular core domain of histone H3 on lysine 56 (H3K56) plays a critical role in chromatin disassembly during transcriptional activation (Williams *et al.*, 2008, Xu *et al.*, 2005) and packing DNA into chromatin following DNA replication and repair in yeast (Chen *et al.*, 2008a, Li *et al.*, 2008, Masumoto *et al.*, 2005). H3K56 acetylation also plays a role in anti-silencing, and is required for transcription at heterochromatin loci (Varv *et al.*, 2010, Xu *et al.*, 2007). H3K56 acetylation in yeast is catalyzed by the fungal-specific histone acetyltransferase (HAT) Rtt109 (Driscoll *et al.*, 2007, Han *et al.*, 2007). *RTT109* expression and H3K56Ac peaks during S phase when new histones are synthesized (Masumoto et al., 2005). H3K56Ac facilitates histone deposition onto replicating DNA and nucleosome assembly (Chen et al., 2008a, Li et al., 2008). Replication-independent dynamic histone exchange has been mapped to promoters and the newly deposited histones are marked by acetylation at H3K56 before deposition (Rufiange *et al.*, 2007, Kaplan *et al.*, 2008, Dion *et al.*, 2007). In yeast H3K56 is deacetylated by

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sirtuin class HDACs, Hst3 and Hst4, with Sir2 contributing some activity (Celic *et al.*, 2006, Maas *et al.*, 2006) (Xu et al., 2007). H3 lysine 56 acetylation marks correlate with developmentally important loci upon human embryonic stem cell differentiation, and co-localizes with key pluripotency regulators (Xie et al., 2009). Recently, fungal Rtt109 was found to be an unexpected structural homolog of metazoan CBP/p300 (Tang *et al.*, 2008), and H3K56 acetylation in multicellular organisms is shown to be catalyzed by CBP/p300 (Das *et al.*, 2009) but competing reports conclude that GCN5/KAT2A is the main acetylase responsible for H3K56Ac in human cells (Tjeertes *et al.*, 2009). p300 was found to map with the core pluripotency regulators in embryonic stem cells by ChIP-seq (Chen *et al.*, 2008b), and *in vivo* mapping of p300 binding sites by ChIP-seq in mouse embryonic tissues has accurately predicted tissue-specific activity of enhancers (Visel *et al.*, 2009, Blow *et al.*, 2010), consistent with the critical roles of p300 in mouse embryonic development (Yao *et al.*, 1998). Significance of H3K56 acetylation in fungal development and in white-opaque cell type specialization in *C. albicans* is not known.

Recent studies have shown that H3K56 acetylation in *C. albicans* is regulated by Rtt109 and Hst3, and both genes are important to maintain genome integrity as in *S. cerevisiae* (Lopes da Rosa *et al.*, 2010, Wurtele *et al.*, 2010). These studies were performed in **a**/α strains, thus the function of *RTT109* and *HST3* in white and opaque cell type formation was not determined. In identifying conditions that promoted white-to-opaque switching, we discovered that nicotinamide (NAM) is a potent stimulator of white-opaque switching. *HST3/hst3* haploinsufficiency in the presence of NAM indicates that nicotinamide targets Hst3. We find that reducing *HST3* copy number also led to an increased frequency of opaque cell formation. We also discovered that genotoxic stress induces white-to-opaque switching by reducing Hst3 level. Unlike all other characterized histone-modifying enzymes (Hnisz et al., 2009), we find that cells lacking *RTT109* have inefficient opaque cell type formation and fail to properly maintain the opaque cell type. Together, our data suggest that *RTT109* and *HST3* genes are important for opaque cell type formation and maintenance. This offers new insight into the basis of cell type formation and maintenance in a unicellular eukaryote.

Results

rtt109 mutants do not efficiently undergo white-to-opaque switching

To investigate the importance of *RTT109* in cell type formation from white-to-opaque, we converted the *rtt109* mutant PKA13 (Lopes da Rosa et al., 2010) from **a**/α mating type to α/α mating type by sorbose selection as described in (Kabir *et al.*, 2005). Quantitative switching assays were performed to determine the frequency of opaque formation from white cells for the *rtt109* mutant. Deletion of *RTT109* resulted in a reduction in spontaneous opaque cell type formation compared to the wild-type α/α strain (Figure 1B). The rare *rtt109* opaque sectors observed do stain with phloxine B indicating genuine opaque cells (Figure 1A). When cells from this opaque sector were plated, only a few opaque-like colonies were observed among mostly white colonies, suggesting that many cells in those opaque sectors were white cells or have reverted to white cells at a high frequency after plating. The lack of opaque colonies and the very low frequency of opaque sectors formed

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from the white cells of the *rtt109* mutant suggest that the H3K56 acetylation or chromatin constructed with H3K56Ac is important for spontaneous white-to-opaque switching.

Spontaneous opaque cell type formation is low in frequency even for wild-type strains under normal growth conditions. To further characterize the importance of *RTT109* in white-to-opaque switching, we performed quantitative switching assays under conditions known to promote white-to-opaque switching. N-acetylglucosamine (GlcNAc) has been shown to be a potent inducer of opaque cell type formation on Lee's media (Huang et al., 2010). Therefore, we performed a quantitative switching assay of wild type and *rtt109* on synthetic complete media, a defined media similar to Lee's media, containing either 2% dextrose (SCD) or 2% GlcNAc (SC-GlcNAc). After 7-days of growth on SC-GlcNAc, 98% of wild-type colonies have efficiently switched, whereas only 10% of *rtt109* colonies switched. (Figure 1C). It should be noted that the fold-increase in GlcNAc stimulated switching for *rtt109* is about 50% of wild type. Longer incubation did not improve the switching frequency for the *rtt109* mutant (data not shown). Growth, as measured by colony size, was not significantly different between SCD and SC-GlcNAc plates for both the wild-type strain and *rtt109* mutants but *rtt109* does grow slower than wild type. This result demonstrates that *RTT109* is important for opaque formation under the GlcNAc induction. *RTT109* is also important for opaque cell type formation under 5% CO_2 (data not shown), a condition reported to promote white-to-opaque switching (Huang et al., 2009). Genotoxins and hydrogen peroxide also induce opaque cell-type formation in *C. albicans* (Alby & Bennett, 2009). The *rtt109* mutant is sensitive to genotoxic and oxidative stress (Lopes da Rosa et al., 2010, Wurtele et al., 2010), so switching could not be reliably assessed under those conditions. However, persistent DNA damage signals exist in the *rtt109* mutant (Lopes da Rosa et al., 2010), yet a high frequency of white-to-opaque switching was not observed in the mutant suggesting that upstream DNA damage signals function through *RTT109* or that opaque cells are not well maintained in the *rtt109* mutant. Together, the quantitative switching assays conducted under various media or environmental conditions identify *RTT109* as a key contributor in opaque cell formation.

Ectopic *WOR1* expression can bypass the requirement for *RTT109* in opaque cell type formation

WOR1 is the master regulator of white-to-opaque switching. Wor1 is necessary to establish and maintain opaque cell types, and ectopic expression of *WOR1* is able to switch white cells opaque on solid media (Huang et al., 2006, Srikantha et al., 2006, Zordan et al., 2006). To determine the genetic relationship between *WOR1* and *RTT109*, a C-terminal 3xHA tagged Wor1 was placed under the glucose-repressible, maltose-inducible *MAL2* promoter (*pMAL2-WOR1-3XHA*). We found that both wild-type cells and *rtt109* cells ectopically expressing *WOR1* switched efficiently after one week (Figure 1D). Wild-type cells with the control vector did not appreciably switch on YEP maltose plates nor did wild-type strains carrying the *MAL2-WOR1-3XHA* transgene switch while grown on YEPD. Our data suggest that Wor1 overexpression could bypass the requirement of *RTT109* in opaque cell formation. However, the extent of opaque cell formation in the *rtt109* mutant may depend on the level of ectopic Wor1 expression, as we had seen reduced switching proficiency in the *rtt109* mutant depending on the induction system and growth media used.

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The *rtt109* mutant is unable to maintain the opaque cell type

To differentiate white and opaque cells at single cell resolution, a reporter gene with GFP under the *WOR1* promoter (Huang et al., 2006) was used to differentiate between white and opaque cells as *WOR1* is highly expressed in opaque cells. Using the reporter gene, we observed *rtt109* opaque cells were slightly larger than wild-type opaque cells (Figure 2A). They also exhibited the typical elongated phenotype of wild type opaque cells but we found that some elongated cells did not express *pWOR1-GFP*, indicating that cell shape alone was not sufficient to distinguish between true opaque form and other morphologies. The reporter gene would also allow us to determine if the level of *WOR1* expression is reduced in the *rtt109* mutant in the opaque cell type, which would be a contributing factor to the opaque cell type establishment and maintenance.

To obtain opaque *rtt109* colonies, cells from the rare opaque sectors were replated onto SCD plates. They grew up as obvious white colonies or opaque colonies that had an atypical phenotype and no white sectors (Figure 2B). The lack of white sectors in those opaque-like colonies and the high percentage of white colonies when replated suggested that each opaque colony might consist of a heterogeneous cell population of both white and opaque cell types. Fluorescent activated cell sorting (FACS) was used to assay *pWOR1-GFP* expression levels in cells collected from several independent colonies (Figure 2B). As determined by FACS, cells from a wild-type opaque colony gave predominantly a single peak that represented similar *WOR1* expression levels within the colony population of cells. In contrast, cells collected from the *rtt109* opaque-like colonies display a wider range of *WOR1* expression levels, and a significant proportion of the cell population had low levels of *WOR1* expression similar to that from white cells (Figure 2B). This was confirmation that cells in individual opaque-like colonies of *rtt109* mutants were heterogeneous in cell types but with no obvious white sectors. We also took *rtt109* opaque cells formed by ectopic expression of *WOR1* and determined that the opaque stability was also not maintained (data not shown). In addition, the percentages of white colonies generated from each of the three independent opaque *rtt109* colonies were consistently higher than the corresponding percentages of cells with low levels of *WOR1* expression by FACS. This suggested that a significant portion of the *WOR1* expressing cells had switched to white cells when plated on SCD during the quantitative switching assay. To explore this observation further, we assayed the stability of the *rtt109* mutant opaque cell type in liquid culture (Figure 2C). Wild-type opaque cells retained a sharp peak of *WOR1* expression characteristic of opaque cells after 24- and 48-hours. The maintenance of the opaque cell type was demonstrated because subsequent plating of liquid grown wild-type cells grew up as opaque colonies. In contrast the *pWOR1-GFP* expression in the *rtt109* opaque population shifted mostly to the level of white cells after 24- and 48-hours. Subsequent plating of the *rtt109* cells after 24- and 48-hours confirmed that there was a rapid loss of the opaque cell type when grown in liquid culture.

Nicotinamide induces white-to-opaque switching in an *RTT109* dependent manner

Based on the impaired switching phenotype of the *rtt109* mutant, we reasoned that increased levels of H3K56Ac might lead to increased spontaneous white-to-opaque switching. Nicotinamide is a potent non-competitive inhibitor of the Sirtuin family of HDACs, and

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treatment of *S. cerevisiae* cells with nicotinamide increases H3K56Ac *in vivo* (Celic et al., 2006, Landry *et al.*, 2000). Treatment of *C. albicans* with nicotinamide, but not nicotinic acid, increased H3K56 acetylation levels (Figure 3A), a finding also made by (Wurtele et al., 2010). Encouraged that nicotinamide increases H3K56Ac *in vivo*, we next examined whether nicotinamide treatment activates *WOR1* expression. Wild-type white cells were grown in liquid SCD in the presence of 25mM nicotinamide at room temperature. After 24-hours they expressed the *pWOR1-GFP* reporter at a level similar to opaque cells, whereas untreated cells did not (Figure 3B). The culture treated with 25mM nicotinamide had a significantly reduced density of cells compared to the untreated culture. When cells from the nicotinamide treated culture were plated, only about 50% of the expected cells plated formed colonies. Within the population of colonies that did grow up, only about 10% were actually opaque. This percentage of opaque colonies was reproducibly higher than cells without nicotinamide treatment, but much lower than expected based on the *pWOR1-GFP* profile. The lower than expected switching frequency could be caused by the inhibition of growth and cell division by nicotinamide or the use of liquid culture to induce switching. Therefore we examined the nicotinamide effect on solid media. Wild-type white cells were plated on SCD agar plates with and without 25mM nicotinamide and incubated at room temperature. 25mM nicotinamide slowed growth significantly, and colonies after one week on nicotinamide were too small to score cell types faithfully. To prevent the agar plates form drying out, test plates were poured thick. After 2-weeks of incubation at room temperature, we noticed that >90% of colonies that grew up formed opaque sectors (Fig. 3C, left panel), but there was about a 10% reduction in cell density compared to untreated cells (data not shown). FACS analysis of cells from the colonies with opaque sectors indicated the presence of opaque cells (Fig. 3C, right panel). This switch was heritable because cells from several independent sectors were plated to SCD plates lacking nicotinamide and a majority of colonies that grew up remained opaque (data not shown). We also titrated nicotinamide concentrations and found that 0.05mM nicotinamide can still induce switching at an appreciable level (data not shown). In accordance with the postulation that chromatin constructed with higher amounts of H3K56Ac is a driver of nicotinamide-induced switching, *rtt109* mutants failed to switch in the presence of nicotinamide (Figure 3C). The growth inhibition by nicotinamide is also completely abolished by the deletion of *RTT109* (data not shown and (Wurtele et al., 2010)).

HST3/hst3 mutants have increased white-to-opaque switching

Nicotinamide most likely can inhibit any sirtuin (Landry et al., 2000). The *C. albicans* genome contains four sirtuin genes: *SIR2*, *HST1*, *HST2*, and *HST3* (Skrzypek *et al.*, 2010). Deletions of *SIR2* or *HST1* did not dramatically increase white-to-opaque switching frequency while deletion of *HST2* reduced the frequency of white-to-opaque switching (Hnisz et al., 2009). *HST3* has not been characterized in white-opaque switching before (Hnisz et al., 2009). In the course of our study Wurtele *et al.* also identified *HST3* as the H3K56 deacetylase in *C. albicans* (Wurtele et al., 2010). In that study, mass spectrometry measured double the amount of H3K56Ac levels in the two independently generated *HST3/hst3* mutants. *HST3* is not an essential gene in *S. cerevisiae*, but *HST3* is an essential gene in *C. albicans*, yet both copies of *HST3* can be deleted in the *rtt109* mutant. We constructed two independent heterozygous *HST3/hst3::FRT* deletion strains designated HLY3993 and

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HLY3994 in the WO-1 background using the Flp/FRT sequence specific recombination system (Reuss *et al.*, 2004) . Subsequently, strain HLY3993 was used to generate HLY4041 (*HST3/hst3::HST3-MYC*), where *HST3* was reintroduced into the disrupted *hst3*::FRT locus. Characterization of Wild-type (TS3.3), *HST3/hst3::FRT* (HLY3993), and *HST3/hst3::HST3-MYC* (HLY4041) strains were carried out. First we tested if nicotinamide targets Hst3 *in vivo* (Figure 4A) by haploinsufficiency for growth in *HST3/hst3* strain (HLY3993), a method used in genome-wide identification of drug targets in *S. cerevisiae* (Giaever *et al.*, 1999). We found the *HST3/hst3* strain was more sensitive to nicotinamide compared to wild-type cells. Restoration of *HST3-MYC* into the *HST3/hst3* strain eliminated the haploinsufficiency. Next we carried out quantitative switching assays in the white-to-opaque and opaque-to-white directions. The heterozygous strain had a 5-fold increase in white-to-opaque switching over the wild-type strain but reintroducing *HST3-MYC* restored switching frequencies of the *HST3/hst3* strain to wild type levels (Figure 4B). This suggests that reducing the copy number of *HST3* from two copies to one copy is sufficient to increase white-to-opaque switching frequency. This result, together with the finding that *rtt109* mutants are insensitive to nicotinamide-induced switching, suggests that increased levels of H3K56Ac increase spontaneous opaque cell-type formation. We did not detect a difference in the opaque-to-white switch between the wild-type and *HST3/hst3* mutants (Figure 4B), indicating additional layers of regulation in opaque cells. Our study identifies a function of *HST3* in regulating switching, most likely via H3K56Ac status.

Genotoxic stress induces white-to-opaque switching by reducing Hst3 level

Methyl methanesulfonate (MMS) and hydroxyurea (HU) are known to induce white-to-opaque switching but the underlying molecular mechanism is unknown. In *S. cerevisiae*, Hst3 is down regulated in response to genotoxic stress (Thaminy *et al.*, 2007). Because tight regulation of H3K56Ac is critical in the DNA damage response in both yeasts, such regulation of Hst3 in response to genotoxic stress may be conserved in *C. albicans* (Celic et al., 2006, Driscoll *et al.*, 2007, Lopes da Rosa et al., 2010, Masumoto *et al.*, 2005, Wurtele et al., 2010). To test this possibility, we tagged Hst3 C-terminus with 13xMyc under the endogenous *HST3* promoter and measured Hst3-Myc levels in cells grown in the presence of MMS or HU. The presence of genotoxins resulted in diminished Hst3-Myc levels within 30-min of treatment with MMS and within 1-hour of treatment with hydroxyurea (Figure 5A). Genotoxin induced loss of Hst3-Myc could be caused by *HST3* transcriptional repression, Hst3 protein stability, or a combination of both. To test if Hst3 protein stability is regulated in response to genotoxins, *HST3-MYC* was placed under the control of the glucose-repressible, maltose-inducible *MAL2* promoter. Hst3-Myc protein stability was determined after *MAL2* promoter shutdown by adding glucose in the presence or absence of genotoxins. MMS treatment resulted in a loss of Hst3-Myc after promoter shutdown, indicating that Hst3 protein was unstable under this condition. Because the dynamics of Hst3-Myc disappearance under its endogenous promoter and the *MAL2* promoter were similar, *HST3* transcription is probably repressed during MMS treatment as well. HU treatment resulted in a gradual loss of Hst3-Myc levels after shutdown, somewhat similar to the no treatment samples (Figure 5B).

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If genotoxic stress induces the white-to-opaque switching through down-regulating *HST3* levels, ectopically expressing *HST3* should render cells insensitive to genotoxic stress in cell type switching, and conversely, the heterozygous *HST3/hst3* mutant is likely to show increased white-to-opaque switching in the presence of genotoxins. To examine the importance of *HST3* regulation by genotoxins in white-opaque switching, quantitative switching assays were performed by growing white cells of the wild type, *HST3/hst3* mutants, and the complemented strain on SCD agar plates containing genotoxins MMS or HU (Figure 5C). Under both conditions tested, *HST3/hst3* mutants had a higher percentage of opaque cell formation than the wild-type control and the complemented strain. The switching difference between *HST3/hst3* baseline vs. genotoxic induced switching is not as dramatic compared to wild-type baseline vs. genotoxin induced switching. This is most likely due to the sensitization of *HST3/hst3* and acquired tolerance to constitutively higher H3K56Ac levels. Ectopic expression of *HST3* under the *MAL2* promoter strongly reduced both MMS and HU induced opaque cell formation (Figure 5D). We observed no significant difference in white-to-opaque switching frequency between wild-type cells in the absence of genotoxins and the *pMAL2*-driven *HST3* strains in the presence of genotoxins. The observation that ectopic expression of *HST3* can suppress genotoxin induced switching strongly suggests that the down regulation of *HST3* transcription and/or Hst3 protein levels is a key regulatory step in response to genotoxin induced switching. These findings demonstrate that genotoxins, Hst3, and switching work in a similar pathway and provide a molecular mechanism for genotoxin induced switching.

H3K56Ac correlates with transcription

We have identified *RTT109* and *HST3* genes as contributing regulators of opaque cell type formation and maintenance. Histone H3K56 is a shared target for acetylation/deacetylation reactions by the encoded enzymes. To investigate whether H3K56Ac exhibits any cell-type specific profile or locus specific enrichment, chromatin cross-linking and immunoprecipitation (ChIP) with anti-H3 and anti-H3K56Ac antibodies was performed in wild-type white and opaque cell types, as well as *rtt109* white cells (Figure 6). Several non-cell type specific and cell-type specific regions were probed for H3K56Ac enrichment. First, an intergenic region (IG) was probed between ORFs 19.3983 and 19.3984, where Wor1 does not bind and the proximal genes are not differentially expressed between wild-type white and opaque cells, nor is expression different between wild type and *rtt109*. This IG region would allow an assessment of H3 and H3K56Ac/H3 levels where Wor1 is absent and no transcription is occurring. Secondly, the *ADE2* +1 position was probed because it is constitutively expressed, does not have differential expression between white and opaque cells, and is not expressed differently between wild type and *rtt109* strains. *WH11* and *OP4* are genes preferentially expressed in stable white and opaque cell types. Thus regions proximal to the +1 position and at the +1 position of each gene were included to assess if H3K56Ac correlated with cell specific expression. Lastly, *EFG1* and *WOR1* are key genes controlling switching and were included to determine if H3K56Ac also correlated with their transcriptional activity. In general, the H3K56Ac/H3 ratio correlated with the transcriptional state of the corresponding gene. We could detect enrichment of H3K56Ac/H3 signal at the IG region, indicating that enrichment occurs in the absence of proximal genes and transcription. The *ADE2* gene is not cell-type regulated. Accordingly, we did not detect a

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difference in H3K56Ac/H3 levels between white and opaque cells. Comparing the H3K56Ac/H3 ratio at *WH11* and *OP4* regions, we could detect a difference that depended on cell type. This difference was more pronounced at *WH11* than *OP4*. The *EFG1* and *WOR1* regions followed a similar profile as was seen in *WH11* and *OP4*, although a difference between white and opaque cells in the H3K56Ac/H3 ratio was not detected at the transcriptional start site of *WOR1*. The ChIP experiments also identified that opaque cells have less chromatin bound H3 across several of the regions tested compared to white cells (Figure 6 lower panel). Consistent with the ChIP H3 data, total cell extract from opaque cells showed a lower level of H3 than white cells (data not shown). Numbers of histone transcripts were found to be fewer in opaque cells in a previous RNA (Tuch et al. 2010).

Discussion

How do unique and heritable phenotypes emerge from the same genotype is a fundamental question in biology. Here we utilized white-opaque switching in *C. albicans* as a simple model to study cell type formation and maintenance. We have shown that deletion of the histone acetyltransferase *RTT109* for H3K56 reduces both spontaneous opaque cell formation and decreases the efficiency of switching in the presence of environmental conditions known to stimulate the white-to-opaque switch. Ectopic expression of *WOR1* can mostly bypass the requirement for the *RTT109* gene. Once opaque, cells lacking *RTT109* cannot maintain the opaque cell type on solid or in liquid culturing conditions. Conversely, reducing the copy number of *HST3* or inhibiting the deacetylase activity by nicotinamide treatment leads to an increase in white-to-opaque switching. Together, these data suggest that modulation of H3K56 acetylation and possibly the subsequent chromatin composition plays a significant role in the opaque cell type formation and maintenance in *C. albicans*. The importance of this finding is further highlighted by the fact that a comprehensive deletion analysis of most known histone modifying enzymes found only a few deletion mutants that showed changes in switching frequency, but none showed an inability to maintain the opaque cell type (Hnisz *et al.*, 2009). Therefore, acetylation of other sites on histone or non-histone proteins by HATs and HDACS, other than Rtt109 and Hst3, do not seem to play such a dramatic role in opaque cell formation and maintenance, although we could not exclude the possibility that Rtt109 and Hst3 regulate the acetylation of a site other than H3K56 that is important for opaque cell formation.

Acetylation of histone H3 lysine 56 is a mark associated with both replication-dependent and replication-independent histone replacement (Rando & Chang, 2010). In *S. cerevisiae*, the replication-independent histone replacement is mapped to promoters of both actively transcribed and repressed genes (Rufiange et al., 2007, Kaplan *et al.*, 2008). Cells lacking H3K56Ac have globally reduced histone turnover and Rtt109 preferentially enhances the histone turnover at rapidly replaced nucleosomes (Kaplan *et al.*, 2008). Dynamic histone turnover in promoter regions may transiently expose DNA to DNA-binding factors and regulate transcription. In addition, rapid histone replacement is found at chromatin boundary elements, suggesting a potential function in preventing the lateral spreading of chromatin states and thereby insulating chromatin domains (Rando & Chang, 2010, Dion et al., 2007, Mito *et al.*, 2007, Dhillon *et al.*, 2009). H3K56 acetylation is also suggested to provide a positive-feedback loop because replacement of one nucleosome enhances subsequent

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nucleosome replacement at the same location (Kaplan et al., 2008). It is possible that the *WOR1* promoter and other Wor1-bound promoters are associated with rapid histone turnover and/or eviction and these events are important for the induction or sustained expression of these genes. Consistent with this notion, we find a general correlation between H3K56Ac enrichment and the transcriptional state of the cell-type specific gene. Interestingly, induction from the *MAL2* promoter is slower in the *rtt109* mutant compared to wild type cells (data not shown). This observation in *C. albicans* is consistent with the reported role of Rtt109 in chromatin disassembly during transcriptional activation (Williams *et al.*, 2008, Xu *et al.*, 2005). It is conceivable that Rtt109 function is a critical part of the Wor1-mediated positive feedback. We expect these events to work in concert with previously recognized transcriptional loops. The expression of other Wor1-regulated white and opaque genes is likely affected in a similar manner.

Histone H3K56 deacetylation is regulated through the sirtuin HDACs Hst3/Hst4 in *S. cerevisiae. HST3* and *HST4* are expressed during the G2 and M phases (Celic *et al.*, 2006, Maas *et al.*, 2006), and their protein levels are down regulated in response to genotoxic stress (Thaminy *et al.*, 2007). Treatment of white cells with nicotinamide increased H3K56Ac levels *in vivo* and activated *WOR1* gene expression in liquid culture. Nicotinamide treatment also stimulated opaque cell formation on agar plates but cells lacking *RTT109* do not switch. This effect of nicotinamide is likely mediated mostly through the inhibition of Hst3 because deletion of one copy of *HST3* is sufficient to increase spontaneous opaque cell formation by 5-fold. In contrast, deletion of other sirtuin genes does not lead to an increase in the frequency of spontaneous opaque cell formation (Hnisz et al., 2009). We further show that genotoxic stress induces white-to-opaque switching by reducing levels of Hst3, and overexpression of *HST3* can suppress genotoxin induced cell type switching. Therefore, DNA damage signaling and Hst3 mediated genome integrity might converge on H3K56Ac status, subsequently leading to switching. On the other hand, *rtt109* has constitutive DNA damage signaling, yet do not switch. This suggests that H3K56Ac is a key effecter subsequent to the upstream DNA damage-signaling pathway responsible for white-to-opaque switching. The signaling pathways responsible for the genotoxin induced Hst3 down-regulation in *C. albicans* have not been defined yet. This could be mediated through the genome integrity checkpoint protein Mec1 kinase as in *S. cerevisiae* (Thaminy et al., 2007), or indirectly through an arrest in cell cycle. In addition to genotoxins, *HST3* transcript level is repressed upon high-level of peroxide stress (Enjalbert *et al.*, 2006), and peroxide treatment of white cells enhances opaque cell type formation (Alby & Bennett, 2009), suggesting that peroxide-induced opaque cell formation may also signal through Hst3. Our results provide molecular connections for the metabolite nicotinamide, genotoxic stress and the sirtuin Hst3 in the epigenetic regulation of white-opaque switching.

Experimental procedures

Yeast Strains and Culturing Conditions

C. albicans strains used in this study are listed in Table 1 as following: *C. albicans* JYC5, an *a/a* mating type derivative from SC5314 (wild-type clinical isolate (Huang et al., 2006)); *C.*

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albicans rtt109 mutant PKA13 (generous gift from Paul Kaufman (Lopes da Rosa et al., 2010)) converted to *a/a* mating type; *C. albicans* TS3.3 derived from WO-1 (generous gift from David Soll (Slutsky et al., 1985, Srikantha *et al.*, 1998)). To propagate strains used in experiments, yeast were routinely grown on SCD or YEPD. The *pWOR1-GFP* reporter gene used was initially constructed in (Huang et al., 2006) but the *SAT1* selectable marker (Reuss et al., 2004) replaced the *URA3* marker to allow for selection in the *rtt109* mutant background. The *pMAL2-WOR1-3XHA* transgene was constructed in two cloning steps. First, a 3xHA tag from *pMG1874* (Gerami-Nejad et al. 2009, obtained from the FGSC) was PCR amplified with (F) 5′-cttctgggtcgtattacACCGGtactggtggtggtcggatccccgggttaattaa-3′ (Age*I* site is capitalized) and (R) 5′-tctagaaggaccacctttgattg-3′, sequentially digested first with Age*I*, then with Bgl*II*, and ligated into an Age*I* site found at the very 3′ end of *WOR1* and the unique Bgl*II* site of the *pTET-WOR1* cassette generated before (Ramírez-Zavala et al., 2008). Then genomic DNA from strain HLY3573 (was used to generate a *MAL2-WOR1* amplicon, containing 550bp of the *MAL2* promoter sequence directly upstream of the entire *WOR1* ORF (Huang et al., 2006) with primers (F) 5′-GTCGACaatgattggtttgatatttttgtctagtac-3′ and (R) 5′-gtatgggtatccagtaccggtgtaatacgaccc-3′. An introduced Sa*lI* site is capitalized in the forward primer. This amplicon was sequentially digested with Age*I* first, then *Sal*I, and ligated into the Sal*I*/Age*I* site of *pTET-WOR1-3xHA* replacing the original copy of *WOR1* with the *MAL2-WOR1* amplicon thereby forming a *MAL2-WOR1-3XHA* transgene. Construction of the *HST3/hst3* mutants (HLY3993 and HLY3994) was carried out with the strategy used in (Reuss et al., 2004). Primers used to amplify flanking *ORF19.1934* (*HST3*) regions to replace the endogenous gene by homologous recombination are: (F) 5′-aataGGGCCCaaggtgtgcaaagtggaaaa-3′ and (R) 5′-ccatCTCGAGggcatactagtgcctgaa-3′ for the left HR region. Capitalized letters denote designed Apa*I* and Xho*I* restriction sites. (F) 5′-ttttCCGCGGgaacctgtggctaaagtgc-3′ and (R) 5′-ctttggttgtaGAGCTCgcggagagacaggaaagaa-3′ for the right HR region. Capitalized letters denote designed *SacII* and *SacI* restriction sites. To generate the *pMAL2* driven Hst3-Myc, a similar cloning strategy was used as describe before in (Wang *et al.*, 2007). Primers used to amplify *ORF19.1934* (*HST3*) are: (F) 5′ gcTCTAGAatgatactgattgacttacttgacaattcagg-3′ and (R) 5′-cgACGCGTatgctgatccaaagtaattcctttctagc-3′. Capitalized letters denote Xba*I* and Mlu*I* restriction sites. A unique Spe*I* restriction site located at the 5′ end of the *HST3* gene allowed for integration of the Hst3-Myc to *HST3* locus, and is under control of the *HST3* promoter. The *HST3/hst3::HST3-MYC* strain was generated by Spe*I* digestion of *pMAL2-HST3-MYC* followed by transformation into *HST3/hst3::FRT* (strain HLY3993). Transformants were screened by PCR for proper integration into the disrupted *hst3:FRT* position. For promoter shutdown and overexpression assays, the *pMAL2-HST3-MYC* cassette was integrated at an *ADE2* gene. All transformations were carried out as described in (Wang et al., 2007).

White–Opaque Switching Assays

C. albicans white–opaque and opaque-white switching assays were performed as reported in (Huang et al., 2006) but synthetic complete or yeast extract peptone (YEP) based media was used instead of Lee's medium. For assays where destination plates were synthetic complete, strains were pre-grown on synthetic complete. For assays where destination plates were YEP based, strains were pre-grown on YEP based media. Strains were routinely grown on

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appropriate solid media for 4-5 days at 25°C before plating to destination plates. Destination plates were: SC 2% dextrose (w/v), SC 2% GlcNAc (w/v), where GlcNAc replaced dextrose, YEP 2% dextrose (w/v) or YEP 2% maltose (w/v), or SC 2% maltose (w/v). Destination plates supplemented with nicotinamide (Sigma-Aldrich), hydroxyurea (Sigma-Aldrich), or methyl methanesulfonate (Sigma-Aldrich) as indicated in the text. Liquid switching assays started off the same as agar based assays but colonies were inoculated into SCD or YEPD media supplemented with nicotinamide (Sigma-Aldrich), or nicotinic acid (Sigma-Aldrich). Subsequent plating was done on SCD agar plates. Routine colony scores were made after one-week incubation at 25°C, or when colonies were large enough to clearly score phenotypes, up to 10 days or two weeks.

FACS analysis

The distribution of fluorescence from the *pWOR1-GFP* reporter gene across the entire cell population was investigated by using a dual laser fluorescence-activated cell sorter (BD FACS Calibur System, Becton Dickinson, San Jose, CA). A minimum of 10,000 cells was routinely measured. The results were analyzed and mean fluorescence was determined with FlowJo Flow Cytometry Analysis Software (version 8.7 for Mac).

Immunoblots

To detect H3K56 acetylation levels wild type white cells were diluted and grown at 30°C in YEPD to an of OD_{600} 0.2 in which 25mM nicotinamide (Sigma-Aldrich) or nicotinic acid (Sigma-Aldrich) was added. After 6 hours protein was extracted as described in (Kushnirov, 2000) and protein levels were probed. H3 protein was detected by rabbit polyclonal antibody, ab1791, as described by the manufacturer (Abcam). H3K56Ac protein was detected by a rabbit monoclonal anti-acetyl-histone H3 clone EPR996Y as described by the manufacture (Millipore). Hst3-Myc was detected by using a horseradish peroxidase-conjugated anti-myc antibody (Roche). Promoter shutdown assays were performed similar to (Wang et al., 2007) except that methyl methanesulfonate and hydroxyurea were added to a final concentration of 0.032% and 40mM respectively. Aliquots were collected after the times indicated, protein was extracted as described in (Kushnirov, 2000) and Hst3-Myc was detected by using a horseradish peroxidase-conjugated anti-myc antibody (Roche). PSTAIRE incubation (Santa Cruz) followed by Goat anti-rabbit conjugated to HRP (Bio rad) was used as a protein loading control.

Chromatin Crosslinking and Immunoprecipitation

ChIP was carried out essentially as described (Hernday *et al.*, 2010). For H3, 2.5ug of rabbit polyclonal antibody, ab1791H3 (Abcam) was incubated with approximately 900ug of crosslinked protein overnight at 4°C. For H3K56Ac, 2ug of rabbit monoclonal anti-acetyl-Histone H3 (Lys56) clone EPR996Y (Millipore) was incubated with approximately 900ug of crosslinked protein overnight at 4°C. Protein A (GE Healthcare) beads were used to for immunoprecipitation. After decrosslinking at 65C° overnight, material was treated with proteinase K (Roche) for 2 hours at 37°C. Protein and nucleic acid material was separated by phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma-Aldrich) extraction followed by overnight ethanol precipitation at -20°C. DNA was quantified using an iQ SYBR green

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Supermix (Bio-Rad) on an iCycler iQ detection system (Bio-Rad). Two independent ChIP experiments were conducted and for each set, the % input of H3 and H3K56Ac/H3 ratio were determined based on Ct values. The ChIP data is the average of these two experiments. The H3 % input is on a log base 2 scale (Log_2). The H3K56Ac/H3 ratio was determined by dividing the % input of H3K56Ac signal by % input of H3 signal. Error bars are standard deviations propagated from the independent ChIP experiments and triplicate QPCR reactions.

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References

Alby K, Bennett RJ. Stress-Induced Phenotypic Switching in Candida albicans. Molecular Biology of the Cell. 2009; 20:3178–3191. [PubMed: 19458191]

Bergen MS, Voss E, Soll DR. Switching at the cellular level in the white-opaque transition of Candida albicans. J Gen Microbiol. 1990; 136:1925–1936. [PubMed: 2269869]

Blow MJ, McCulley DJ, Li Z, Zhang T, Akiyama JA, Holt A, Plajzer-Frick I, Shoukry M, Wright C, Chen F, Afzal V, Bristow J, Ren B, Black BL, Rubin EM, Visel A, Pennacchio LA. ChIP-Seq identification of weakly conserved heart enhancers. Nat Genet. 2010; 42:806–810. [PubMed: 20729851]

Celic I, Masumoto H, Griffith WP, Meluh P, Cotter RJ, Boeke JD, Verreault A. The Sirtuins Hst3 and Hst4p Preserve Genome Integrity by Controlling Histone H3 Lysine 56 Deacetylation. Current Biology. 2006; 16:1280–1289. [PubMed: 16815704]

Chen CC, Carson JJ, Feser J, Tamburini B, Zabaronick S, Linger J, Tyler JK. Acetylated Lysine 56 on Histone H3 Drives Chromatin Assembly after Repair and Signals for the Completion of Repair. Cell. 2008a; 134:231–243. [PubMed: 18662539]

Chen X, Xu H, Yuan P, Fang F, Huss M, Vega VB, Wong E, Orlov YL, Zhang W, Jiang J, Loh YH, Yeo HC, Yeo ZX, Narang V, Govindarajan KR, Leong B, Shahab A, Ruan Y, Bourque G, Sung WK, Clarke ND, Wei CL, Ng HH. Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. Cell. 2008b; 133:1106–1117. [PubMed: 18555785]

Das C, Lucia MS, Hansen KC, Tyler JK. CBP/p300-mediated acetylation of histone H3 on lysine 56. Nature. 2009; 459:113–117. [PubMed: 19270680]

Dhillon N, Raab J, Guzzo J, Szyjka SJ, Gangadharan S, Aparicio OM, Andrews B, Kamakaka RT. DNA polymerase epsilon, acetylases and remodellers cooperate to form a specialized chromatin structure at a tRNA insulator. EMBO J. 2009; 28:2583–2600. [PubMed: 19629037]

Dion MF, Kaplan T, Kim M, Buratowski S, Friedman N, Rando OJ. Dynamics of Replication-Independent Histone Turnover in Budding Yeast. Science. 2007; 315:1405–1408. [PubMed: 17347438]

Driscoll R, Hudson A, Jackson SP. Yeast Rtt109 Promotes Genome Stability by Acetylating Histone H3 on Lysine 56. Science. 2007; 315:649–652. [PubMed: 17272722]

Enjalbert B, Smith DA, Cornell MJ, Alam I, Nicholls S, Brown AJ, Quinn J. Role of the Hog1 stress-activated protein kinase in the global transcriptional response to stress in the fungal pathogen Candida albicans. Mol Biol Cell. 2006; 17:1018–1032. [PubMed: 16339080]

Giaever G, Shoemaker DD, Jones TW, Liang H, Winzeler EA, Astromoff A, Davis RW. Genomic profiling of drug sensitivities via induced haploinsufficiency. Nat Genet. 1999; 21:278–283. [PubMed: 10080179]

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Han J, Zhou H, Horazdovsky B, Zhang K, Xu RM, Zhang Z. Rtt109 Acetylates Histone H3 Lysine 56 and Functions in DNA Replication. Science. 2007; 315:653–655. [PubMed: 17272723]

Hernday AD, Noble SM, Mitrovich QM, Johnson AD. Genetics and molecular biology in Candida albicans. Methods Enzymol. 2010; 470:737–758. [PubMed: 20946834]

Hnisz D, Schwarzmüller T, Kuchler K. Transcriptional loops meet chromatin: a dual-layer network controls white–opaque switching in Candida albicans. Molecular Microbiology. 2009; 74:1–15. [PubMed: 19555456]

Huang G, Srikantha T, Sahni N, Yi S, Soll DR. CO2 Regulates White-to-Opaque Switching in Candida albicans. Current Biology. 2009; 19:330–334. [PubMed: 19200725]

Huang G, Wang H, Chou S, Nie X, Chen J, Liu H. Bistable expression of WOR1, a master regulator of white–opaque switching in Candida albicans. Proceedings of the National Academy of Sciences. 2006; 103:12813–12818.

Huang G, Yi S, Sahni N, Daniels KJ, Srikantha T, Soll DR. N-Acetylglucosamine Induces White to Opaque Switching, a Mating Prerequisite in Candida albicans. PLoS Pathog. 2010; 6:e1000806–e1000806. [PubMed: 20300604]

Kabir MA, Ahmad A, Greenberg JR, Wang YK, Rustchenko E. Loss and gain of chromosome 5 controls growth of Candida albicans on sorbose due to dispersed redundant negative regulators. Proceedings of the National Academy of Sciences of the United States of America. 2005; 102:12147–12152. [PubMed: 16099828]

Kaplan T, Liu CL, Erkmann JA, Holik J, Grunstein M, Kaufman PD, Friedman N, Rando OJ. Cell Cycle- and Chaperone-Mediated Regulation of H3K56ac Incorporation in Yeast. PLoS Genet. 2008; 4:e1000270–e1000270. [PubMed: 19023413]

Kushnirov VV. Rapid and reliable protein extraction from yeast. Yeast. 2000; 16:857–860. [PubMed: 10861908]

Kvaal C, Lachke SA, Srikantha T, Daniels K, McCoy J, Soll DR. Misexpression of the opaque-phase-specific gene PEP1 (SAP1) in the white phase of Candida albicans confers increased virulence in a mouse model of cutaneous infection. Infect Immun. 1999; 67:6652–6662. [PubMed: 10569787]

Lan CY, Newport G, Murillo LA, Jones T, Scherer S, Davis RW, Agabian N. Metabolic specialization associated with phenotypic switching in Candidaalbicans. Proceedings of the National Academy of Sciences of the United States of America. 2002; 99:14907–14912. [PubMed: 12397174]

Landry J, Slama JT, Sternglanz R. Role of NAD+ in the Deacetylase Activity of the SIR2-like Proteins. Biochemical and Biophysical Research Communications. 2000; 278:685–690. [PubMed: 11095969]

Li Q, Zhou H, Wurtele H, Davies B, Horazdovsky B, Verreault A, Zhang Z. Acetylation of Histone H3 Lysine 56 Regulates Replication-Coupled Nucleosome Assembly. Cell. 2008; 134:244–255. [PubMed: 18662540]

Lohse MB, Johnson AD. Differential Phagocytosis of White versus Opaque Candida albicans by Drosophila and Mouse Phagocytes. PLoS ONE. 2008; 3:e1473–e1473. [PubMed: 18213381]

Lopes da Rosa J, Boyartchuk VL, Zhu LJ, Kaufman PD. Histone acetyltransferase Rtt109 is required for Candida albicans pathogenesis. Proceedings of the National Academy of Sciences. 2010; 107:1594–1599.

Maas NL, Miller KM, DeFazio LG, Toczyski DP. Cell Cycle and Checkpoint Regulation of Histone H3 K56 Acetylation by Hst3 and Hst4. Molecular Cell. 2006; 23:109–119. [PubMed: 16818235]

Masumoto H, Hawke D, Kobayashi R, Verreault A. A role for cell-cycle-regulated histone H3 lysine 56 acetylation in the DNA damage response. Nature. 2005; 436:294–298. [PubMed: 16015338]

Miller MG, Johnson AD. White-Opaque Switching in Candida albicans Is Controlled by Mating-Type Locus Homeodomain Proteins and Allows Efficient Mating. Cell. 2002; 110:293–302. [PubMed: 12176317]

Mito Y, Henikoff JG, Henikoff S. Histone replacement marks the boundaries of cis-regulatory domains. Science. 2007; 315:1408–1411. [PubMed: 17347439]

Morrow B, Anderson J, Wilson J, Soll DR. Bidirectional stimulation of the white-opaque transition of Candida albicans by ultraviolet irradiation. J Gen Microbiol. 1989; 135:1201–1208. [PubMed: 2695598]

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Morschhauser J. Regulation of white-opaque switching in Candida albicans. Med Microbiol Immunol. 2010; 199:165–172. [PubMed: 20390300]

Ramírez-Zavala B, Reuβ O, Park YN, Ohlsen K, Morschhäuser J. Environmental Induction of White–Opaque Switching in Candida albicans. PLoS Pathog. 2008; 4:e1000089–e1000089. [PubMed: 18551173]

Rando OJ, Chang HY. Genome-Wide Views of Chromatin Structure. Annu Rev Biochem. 2010; 78:245–271. [PubMed: 19317649]

Reuss O, Vik A, Kolter R, Morschhäuser J. The SAT1 flipper, an optimized tool for gene disruption in Candida albicans. Gene. 2004; 341:119–127. [PubMed: 15474295]

Rikkerink EH, Magee BB, Magee PT. Opaque-white phenotype transition: a programmed morphological transition in Candida albicans. J Bacteriol. 1988; 170:895–899. [PubMed: 2828333]

Rufiange A, Jacques Pt, Bhat W, Robert Fo, Nourani A. Genome-Wide Replication-Independent Histone H3 Exchange Occurs Predominantly at Promoters and Implicates H3 K56 Acetylation and Asf1. Molecular Cell. 2007; 27:393–405. [PubMed: 17679090]

Skrzypek MS, Arnaud MB, Costanzo MC, Inglis DO, Shah P, Binkley G, Miyasato SR, Sherlock G. New tools at the Candida Genome Database: biochemical pathways and full-text literature search. Nucleic Acids Research. 2010; 38:D428–D432. [PubMed: 19808938]

Slutsky B, Buffo J, Soll DR. High-frequency switching of colony morphology in Candida albicans. Science. 1985; 230:666–669. [PubMed: 3901258]

Slutsky B, Staebell M, Anderson J, Risen L, Pfaller M, Soll DR. "White-opaque transition": a second high-frequency switching system in Candida albicans. The Journal of Bacteriology. 1987; 169:189–197.

Srikantha T, Borneman AR, Daniels KJ, Pujol C, Wu W, Seringhaus MR, Gerstein M, Yi S, Snyder M, Soll DR. TOS9 Regulates White-Opaque Switching in Candida albicans. Eukaryotic Cell. 2006; 5:1674–1687. [PubMed: 16950924]

Srikantha T, Tsai L, Daniels K, Enger L, Highley K, Soll DR. The two-component hybrid kinase regulator CaNIKl of Candida albicans. Microbiology. 1998; 144:2715–2729. [PubMed: 9802013]

Srikantha T, Tsai L, Daniels K, Klar AJS, Soll DR. The Histone Deacetylase Genes HDA1 and RPD3 Play Distinct Roles in Regulation of High-Frequency Phenotypic Switching in Candida albicans. The Journal of Bacteriology. 2001; 183:4614–4625.

Srikantha T, Tsai LK, Daniels K, Soll DR. EFG1 Null Mutants of Candida albicans Switch but Cannot Express the Complete Phenotype of White-Phase Budding Cells. The Journal of Bacteriology. 2000; 182:1580–1591.

Tang Y, Holbert MA, Wurtele H, Meeth K, Rocha W, Gharib M, Jiang E, Thibault P, Verreault A, Cole PA, Marmorstein R. Fungal Rtt109 histone acetyltransferase is an unexpected structural homolog of metazoan p300/CBP. Nat Struct Mol Biol. 2008; 15:998–998.

Thaminy S, Newcomb B, Kim J, Gatbonton T, Foss E, Simon J, Bedalov A. Hst3 Is Regulated by Mec1-dependent Proteolysis and Controls the S Phase Checkpoint and Sister Chromatid Cohesion by Deacetylating Histone H3 at Lysine 56. Journal of Biological Chemistry. 2007; 282:37805–37814. [PubMed: 17977840]

Tjeertes JV, Miller KM, Jackson SP. Screen for DNA-damage-responsive histone modifications identifies H3K9Ac and H3K56Ac in human cells. EMBO J. 2009; 28:1878–1889. [PubMed: 19407812]

Tsong AE, Miller MG, Raisner RM, Johnson AD. Evolution of a combinatorial transcriptional circuit: a case study in yeasts. Cell. 2003; 115:389–399. [PubMed: 14622594]

Tuch BB, Mitrovich QM, Homann OR, Hernday AD, Monighetti CK, De La Vega FM, Johnson AD. The Transcriptomes of Two Heritable Cell Types Illuminate the Circuit Governing Their Differentiation. PLoS Genet. 2010; 6:e1001070–e1001070. [PubMed: 20808890]

Varv S, Kristjuhan K, Peil K, Looke M, Mahlakoiv T, Paapsi K, Kristjuhan A. Acetylation of H3 K56 is required for RNA polymerase II transcript elongation through heterochromatin in yeast. Mol Cell Biol. 2010; 30:1467–1477. [PubMed: 20065036]

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Visel A, Blow MJ, Li Z, Zhang T, Akiyama JA, Holt A, Plajzer-Frick I, Shoukry M, Wright C, Chen F, Afzal V, Ren B, Rubin EM, Pennacchio LA. ChIP-seq accurately predicts tissue-specific activity of enhancers. Nature. 2009; 457:854–858. [PubMed: 19212405]

Wang A, Lane S, Tian Z, Sharon A, Hazan I, Liu H. Temporal and Spatial Control of HGC1 Expression Results in Hgc1 Localization to the Apical Cells of Hyphae in Candida albicans. Eukaryotic Cell. 2007; 6:253–261. [PubMed: 17172437]

Williams SK, Truong D, Tyler JK. Acetylation in the globular core of histone H3 on lysine-56 promotes chromatin disassembly during transcriptional activation. Proceedings of the National Academy of Sciences. 2008; 105:9000–9005.

Wurtele H, Tsao S, Lepine G, Mullick A, Tremblay J, Drogaris P, Lee EH, Thibault P, Verreault A, Raymond M. Modulation of histone H3 lysine 56 acetylation as an antifungal therapeutic strategy. Nat Med. 2010; 16:774–780. [PubMed: 20601951]

Xie W, Song C, Young NL, Sperling AS, Xu F, Sridharan R, Conway AE, Garcia BA, Plath K, Clark AT, Grunstein M. Histone H3 Lysine 56 Acetylation Is Linked to the Core Transcriptional Network in Human Embryonic Stem Cells. Molecular Cell. 2009; 33:417–427. [PubMed: 19250903]

Xu F, Zhang K, Grunstein M. Acetylation in Histone H3 Globular Domain Regulates Gene Expression in Yeast. Cell. 2005; 121:375–385. [PubMed: 15882620]

Xu F, Zhang Q, Zhang K, Xie W, Grunstein M. Sir2 Deacetylates Histone H3 Lysine 56 to Regulate Telomeric Heterochromatin Structure in Yeast. Molecular Cell. 2007; 27:890–900. [PubMed: 17889663]

Yao TP, Oh SP, Fuchs M, Zhou ND, Ch'ng LE, Newsome D, Bronson RT, Li E, Livingston DM, Eckner R. Gene dosage-dependent embryonic development and proliferation defects in mice lacking the transcriptional integrator p300. Cell. 1998; 93:361–372. [PubMed: 9590171]

Zordan RE, Galgoczy DJ, Johnson AD. Epigenetic properties of white–opaque switching in Candida albicans are based on a self-sustaining transcriptional feedback loop. Proceedings of the National Academy of Sciences. 2006; 103:12807–12812.

Zordan RE, Miller MG, Galgoczy DJ, Tuch BB, Johnson AD. Interlocking Transcriptional Feedback Loops Control White-Opaque Switching in Candida albicans. PLoS Biol. 2007; 5:e256–e256. [PubMed: 17880264]

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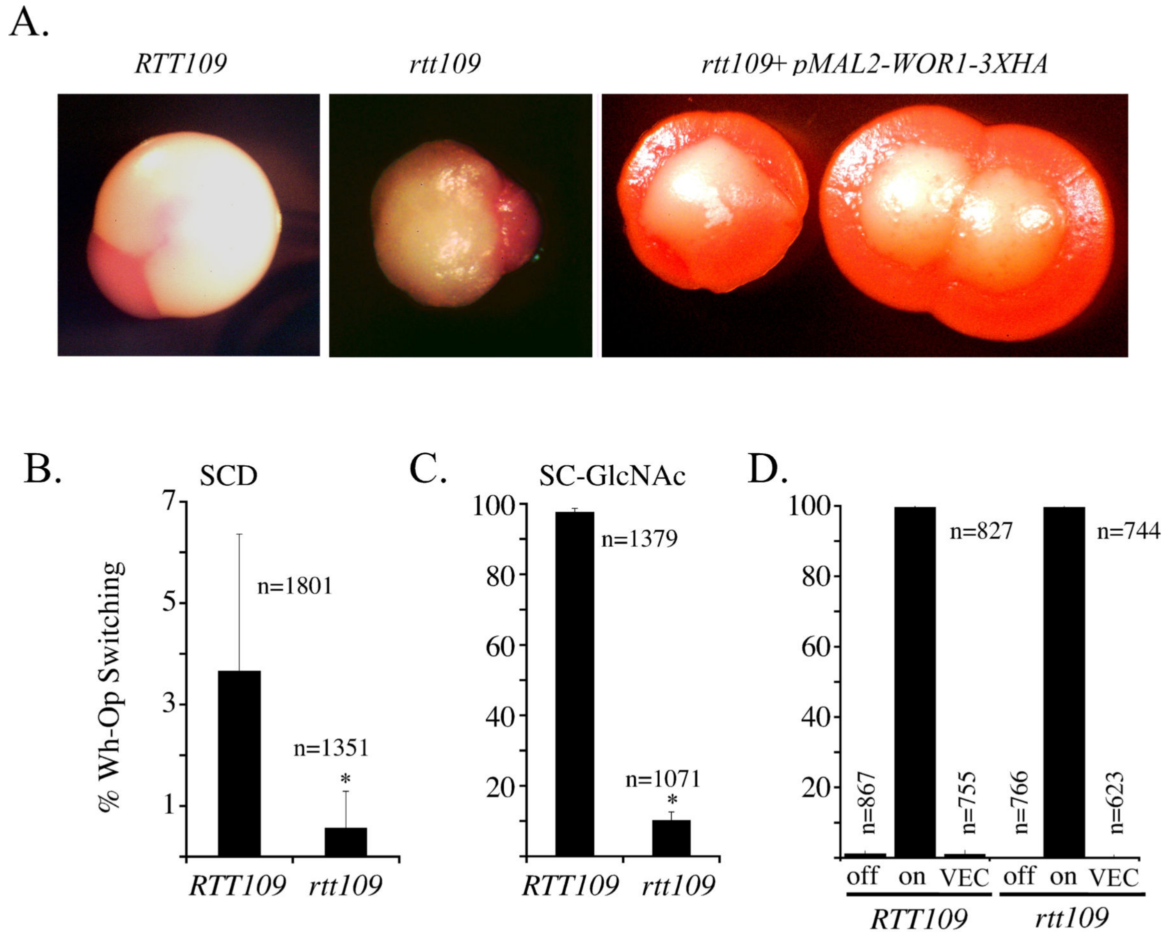

Figure 1. *rtt109* mutants are impaired in white-to-opaque switching

(A) Colony morphology of wild type (HLY3555) and *rtt109* (HLY3997) white colonies with spontaneous opaque sectors on SCD media, and *rtt109* (HLY4057) ectopically expressing *WOR1*. 5 μg/ml phloxine B is used in the plates. (B) Quantitative switching assay on SCD media of wild type (HLY3555) and *rtt109* (HLY3997). (C) Quantitative switching assay on SC+2%GlcNAc media of wild type (HLY3555) and *rtt109* (HLY3997) to determine GlcNAc stimulated white-opaque switching. (D) Quantitative switching assay when ectopically expressing *WOR1*. "off" corresponds to wild type (HLY4055) and *rtt109* (HLY4057) strains grown on YEP+2% dextrose while "on" corresponds to growth on YEP 2% maltose. "VEC" refers to wild type (HLY4025) and *rtt109* (HLY4027) containing the control vector grown on YEP+2% maltose. Data is from at least two independent quantitative switching assays started on different days with different starter cultures. Abbreviations: Wh, white; Op, opaque; SCD, synthetic complete 2% dextrose; GlcNAc, N-acetylglucosamine; n are the total number of colonies scored; error bars are differences in phenotypic scores between replicate tests; * is p-value < 0.01 compared to wild type.

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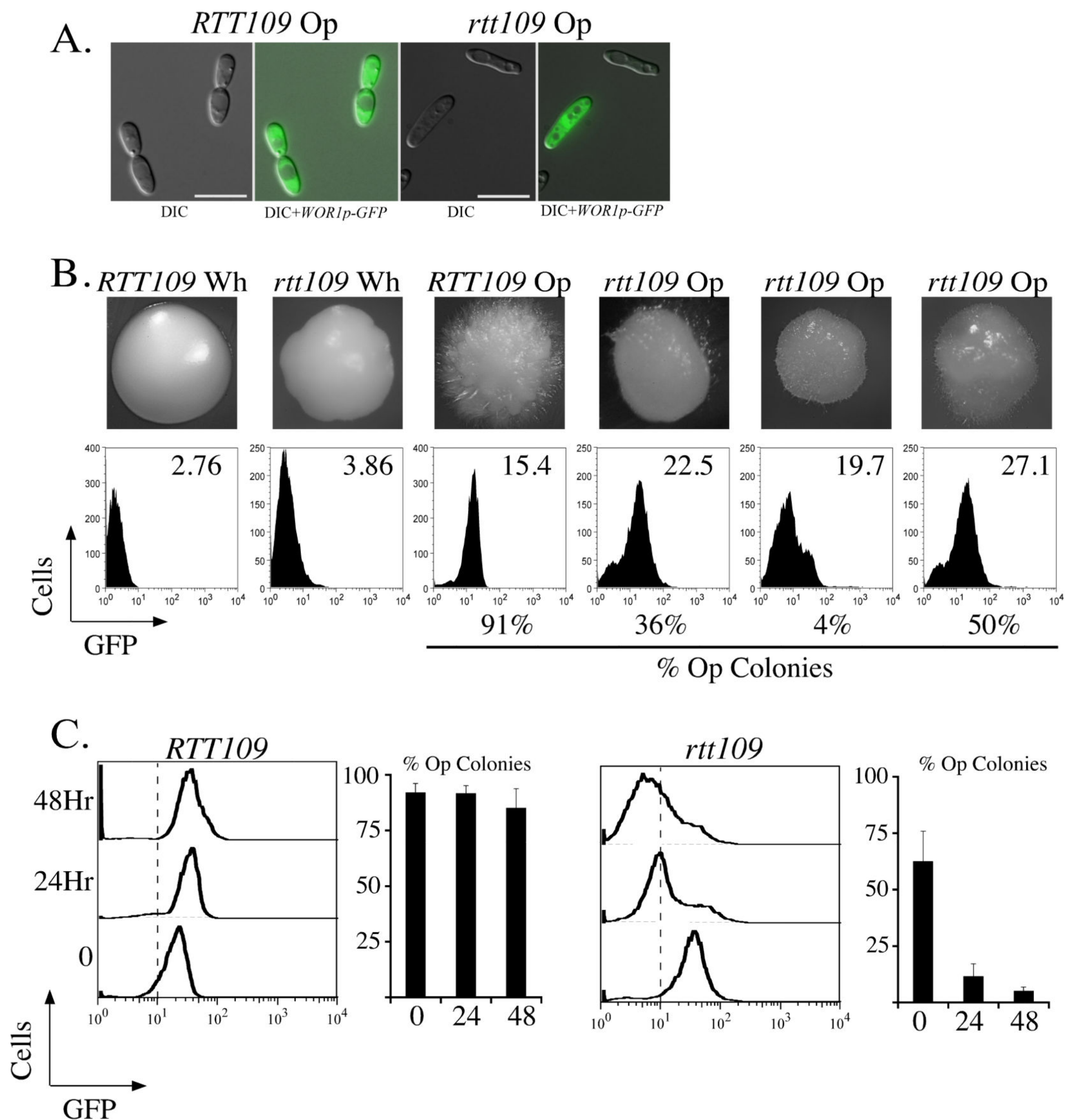

Figure 2. Maintenance of the opaque cell type is lost in the *rtt109* mutant

(A) Wild type (HLY3555) and *rtt109* (HLY3998) opaque cells expressing the *pWOR1-GFP* reporter gene. Scale bar is 10 micrometers. (B) Colony phenotypes and whole colony FACS plots of *pWOR1-GFP* fluorescence from wild type (HLY3555) and *rtt109* (HLY3998) opaque strains. Mean GFP signals are shown in the FACS plots. The % Op colonies that originated from the same colonies used in FACS analysis are indicated. Approximately 500 colonies were scored from each opaque colony for wild type and *rtt109*. (C) *rtt109* opaque cells are unstable during liquid growth. At indicated times, aliquots of cells were removed for FACS analysis and plating assays. Adjoining graphs are colony phenotypes scored 3-5 days after plating. For (C) Wild type: 0-Hr n=1242, 24-Hr n=1463, 48-Hr n=1838. *rtt109*: 0-Hr n=577, 24-Hr n=1101, 48-Hr n=1346. error bars are differences in phenotypic scores between at least two independent tests. Dashed lines in FACS plots for (C) roughly demarcate white and opaque cell types based on fluorescent signal.

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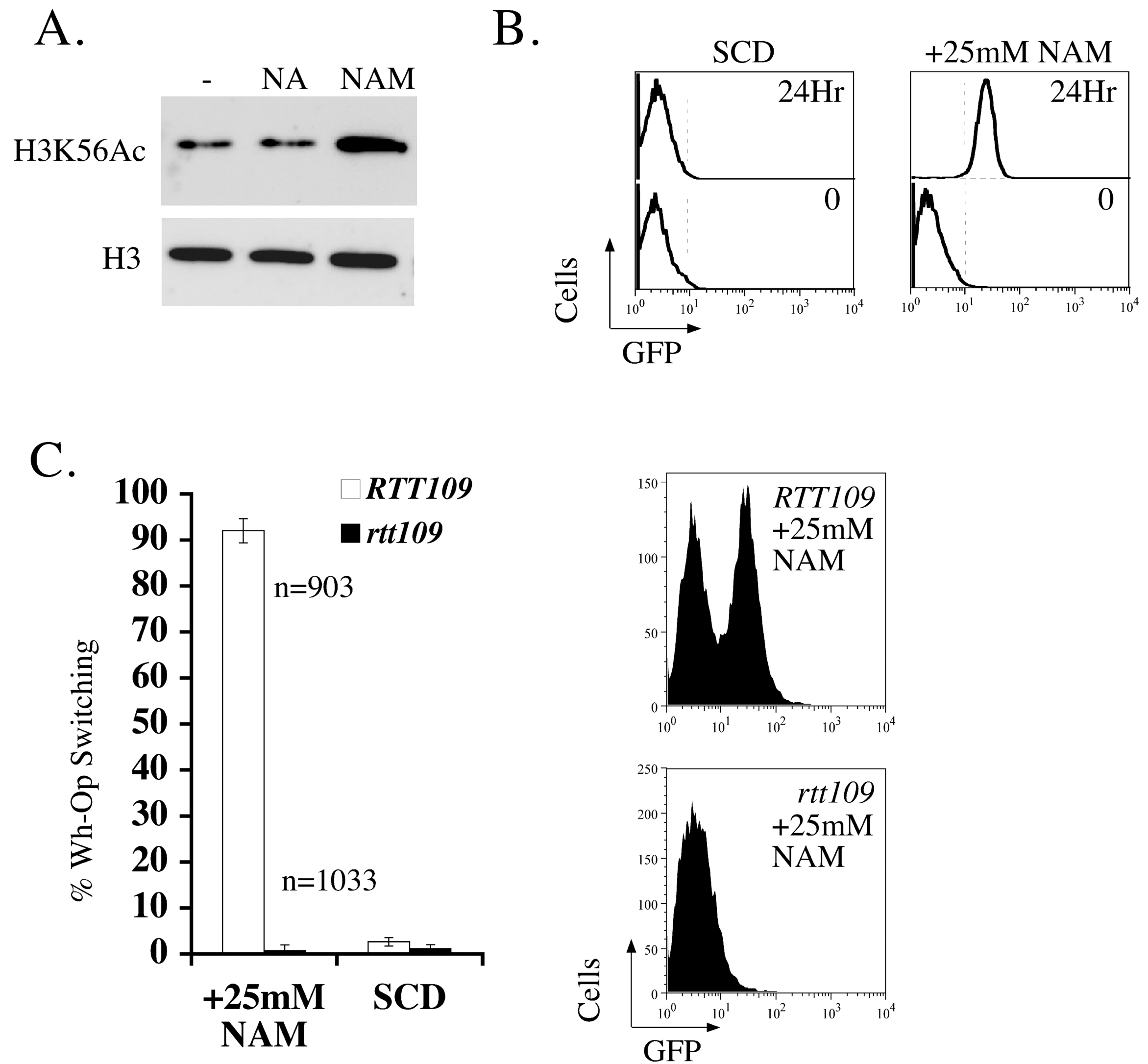

Figure 3. *RTT109* is required for nicotinamide induced switching

(A) Western blot of wild-type white cells (HLY3879) untreated, nicotinic acid treated (25mM), and nicotinamide (25mM) treated. H3K56Ac and H3 protein levels were probed after approximately six hours of growth at 30°C in YEPD media. (B) FACS plots of wild-type white cells grown in liquid SCD culture in the presence or absence of 25mM nicotinamide. (C) Quantitative switching assay of wild type (HLY3555) and *rtt109* (HLY3998) in the presence of 25mM nicotinamide. Adjoining FACS plots are of representative nicotinamide treated colonies of either wild type (HLY3555) or *rtt109* (HLY3998). For figure (C), the SCD without NAM switching frequency data are from Figure 1B with wild type n=1801 and *rtt109* n=1351. Switching data are from at least two independent tests. n are the total number of colonies counted; error bars are differences in phenotypic scores between tests. Dashed lines are added for clarity.

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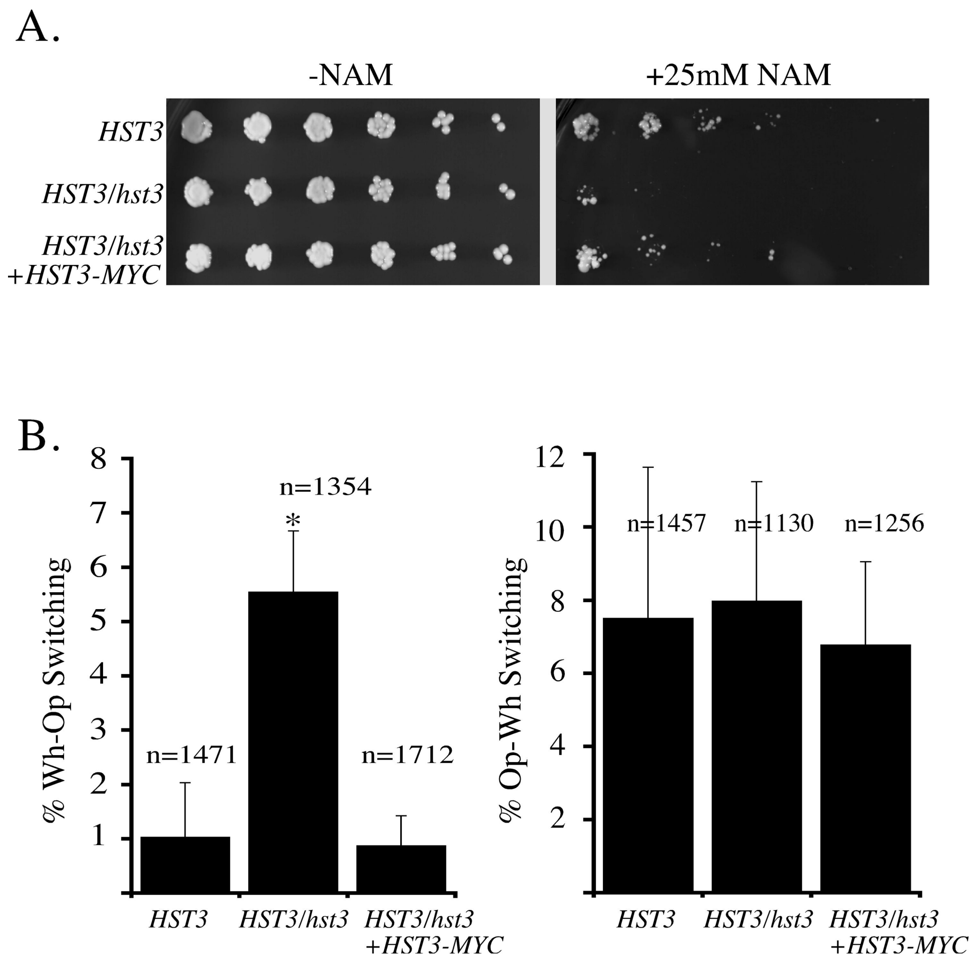

Figure 4. *HST3/hst3* mutants have increased white-opaque switching

(A) Overnight cultures of *HST3/HST3* (TS3.3), *HST3/hst3* (HLY3993), *HST3/hst3::HST3-MYC* (HLY4041) were adjusted to a final OD_{600} of 0.1, after which five 5-fold serial dilutions were spotted onto SCD with or without 25mM nicotinamide and incubated at 30°C for 4 days. (B) Quantitative switching assays of indicated strains were carried out on SCD to determine the switching phenotypes in the white-to-opaque and opaque-to-white directions. Data are from at least two independent tests; n is the total number of colonies counted; error bars are differences in phenotypic scores between tests. * is p-value < 0.01 compared to wild type.

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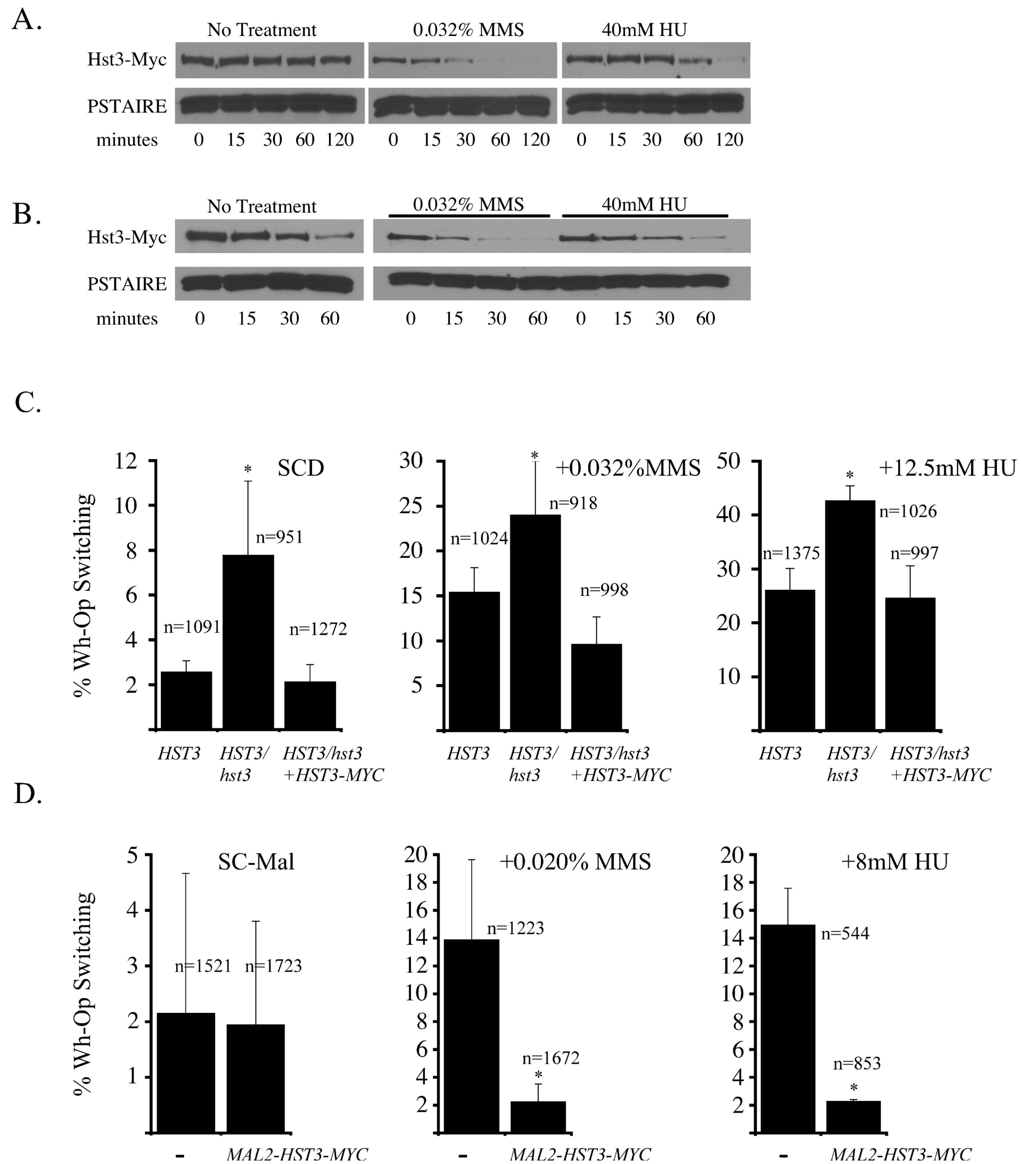

Figure 5. Genotoxins, Hst3, and switching are linked

(A) Western blot of Hst3-Myc from wild type (HLY3995) cultures treated with or without genotoxins for the indicated times. (B) Western blot of Hst3-Myc after *MAL2* promoter shutdown in the presence or absence of genotoxins, strain (HLY3996). PSTAIRE serves as a loading control. (C) Quantitative white-opaque switching assays of wild type (TS3.3), *HST3/hst3* (HLY3993), and *HST3/hst3::HST3-MYC* (HLY4041) in the presence of genotoxins. (D) Quantitative white-opaque switching assays on SC maltose agar plates of wild type (TS3.3) and cells ectopically expressing *HST3* from the *MAL2* promoter strain (HLY3996). SCD is synthetic complete media 2% dextrose. SC-Mal is synthetic complete media 2% maltose. Data are from at least two separate tests; n is the total number of colonies counted; error bars are differences in phenotypic scores between tests. * is p-value < 0.01 compared to wild type.

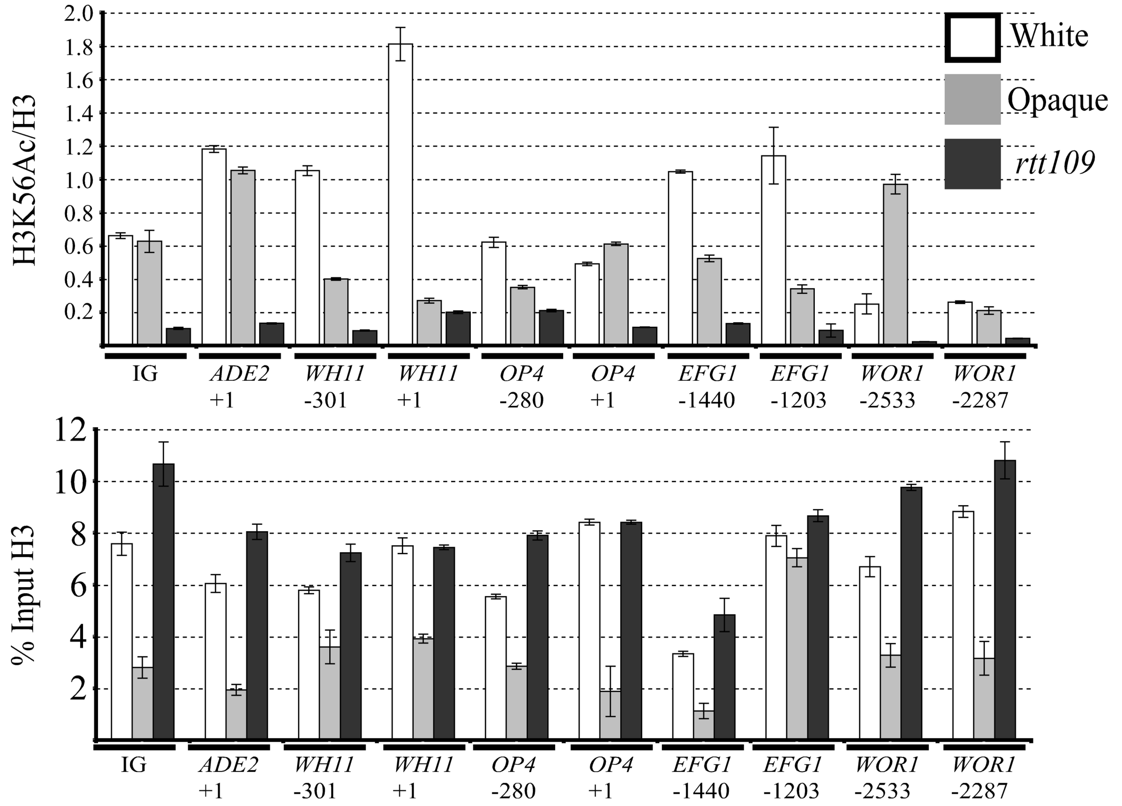

Figure 6. H3K56Ac correlates with cell-type specific transcription

ChIP of H3 and H3K56Ac in wild type (HLY3555) white, opaque, and *rtt109* (HLY3998) white cells. The lower panel is the % input of H3 presented on Log_2 scale. The upper panel is the ratio of the % input for individual H3 and H3K56Ac IP. Data presented is the average of two separate ChIP experiments. Error bars shown are propagated from separate ChIP experiments and triplicate reactions in QPCR reactions. The *rtt109* strain also serves as a negative control for H3K56Ac enrichment. Numbers below gene names are relative to the +1 position, and are in basepair (bp) units. Amplicon lengths extend 5′-3′ direction approximately 100-250bp from the indicated positions, except for the +1 amplicons, where the +1 position is located in the middle portion of the amplicon. Note, *EFG1* and *WOR1* contain large 5′-UTRs, and transcription starts at -1173 and -2000 relative to their +1 position, respectively.

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Table1

Strains used in this study

Strain	Parental Strain	Genotype	Source or reference
TS3.3	Red3/6	*MTLa/α ade2/ade2* *ura3::ADE2/ura3::ADE2*	(Srikantha *et al.*, 2000)
HLY3879	TS3.3	*MTLa/α ade2/ade2* *ura3::ADE2/ura3::ADE2* *WOR1/WOR1/*P_{WOR1}*-GFP-URA3-WOR1*	This study
HLY3993	TS3.3	*MTLa/α ade2/ade2* *ura3::ADE2/ura3::ADE2* *HST3/hst3::FRT*	This study
HLY3994	TS3.3	*MTLa/α ade2/ade2* *ura3::ADE2/ura3::ADE2* *HST3/hst3::FRT*	This study
HLY3995	HLY3993	*MTLa/α ade2/ade2* *ura3::ADE2/ura3::ADE2* *HST3-MYC-URA3-MAL2-HST3/hst1::FRT*	This study
HLY3996	TS3.3	*MTLa/α ade2/ade2* *ura3::ADE2/ura3::ADE2* P_{MAL2}*-HST3-MYC-URA3*	This study
HLY4041	HLY3993	*MTLa/α ade2/ade2* *ura3::ADE2/ura3::ADE2* *HST3/hst3::HST3-MYC*	This study
HLY3555	JYC5	*MTL* ***a/a*** *ura3::imm434/ura3::imm434* *WOR1/*P_{WOR1}*-GFP-URA3-WOR1*	(Huang *et al.*, 2006)
HLY4025	HLY3555	*MTL* ***a/a*** *ura3::imm434/ura3::imm434* *WOR1/*P_{WOR1}*-GFP-URA3-WOR1* *ADH1/adh1::*P_{TET}*-GFP-SAT1*	This study
HLY4055	HLY3555	*MTL* ***a/a*** *ura3::imm434/ura3::imm434* *WOR1/*P_{WOR1}*-GFP-URA3-WOR1* *ADH1/adh1::*P_{MAL2}*-WOR1-3XHA-SAT1*	This study
PKA13	SC5314	*MTL* ***a****/α rtt109::FRT/rtt109::FRT*	(Lopes da Rosa *et al.*, 2010)
HLY3997	PKA13	*MTLa/α rtt109::FRT/rtt109::FRT*	This study
HLY3998	HLY3997	*MTLa/α rtt109::FRT/rtt109::FRT* *WOR1/*P_{WOR1}*-GFP-SAT1-WOR1*	This study
HLY4027	HLY3997	*MTLa/α rtt109::FRT/rtt109::FRT* *ADH1/adh1::*P_{TET}*-GFP-SAT1*	This study
HLY4057	HLY3997	*MTLa/α rtt109::FRT/rtt109::FRT* *ADH1/adh1::*P_{MAL2}*-WOR1-3XHA-SAT1*	This study